Exhibit F

Advances and Challenges in Basic and Translational Research on Clusterin

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Introduction

Clusterin (CLU), known by several other names, has recently drawn much attention because of its association with cancer promotion and metastasis (1–3). First discovered as serum apolipoprotein J with chaperoning properties for protein stabilization, CLU also can exist within the cell to function in either proapoptotic or prosurvival processes. This diverse set of functions can be attributed to the existence of two alternatively spliced forms of the *CLU* gene that encode secretory CLU (sCLU) or nuclear CLU (nCLU). The sCLU form is cytoprotective and inhibiting its prosurvival function is the basis of current phase I/II clinical trials against prostate, lung, and breast cancers, where synergy with various cytotoxic agents has been reported (4). In contrast, nCLU migrates to the nucleus on cytotoxic stress to trigger cell death (5, 6). It interacts with the DNA double-strand break repair antigen Ku70, blocking its function and causing cell death (6).

The 5th International Clusterin Workshop, held in Spetses, Greece on June 2–5, 2008, brought together researchers active in this field to focus on clarifying the mechanisms of this unique protein for executing the fate of a cell. Based on new findings presented, a full debate on whether CLU has a key role in tumor prevention or tumor promotion led to some consensus that it may depend on the context of its expression and the microenvironment.

CLU as a Cell Death/Survival Determinant

The intracellular localization of CLU protein isoforms and the effect on cell survival was an active area of research. Dr. Arkadiusz Orzechowski (Polish Academy of Sciences, Warsaw, Poland) reported decreased nCLU levels with increased sCLU levels *in vitro* or *in vivo* in colorectal cancers in comparison with normal cells/tissues. Importantly, the sCLU:nCLU ratio was a key factor in COLO 205 colon tumor cell survival. These data confirmed previous findings in human colon carcinoma (1). Similarly, Dr. Leonardo Fabbri (University of Modena and Reggio Emilia, Modena, Italy) evaluated CLU isoform expression in human normal bronchial (BEAS-2B) epithelial and A549 lung adenocarcinoma cells after cigarette smoke extract exposure. Again, the sCLU:nCLU ratio seems to be an important determinant of cell survival. Dr. Evangelos Kolettas (University of Ioannina, Ioannina, Greece) analyzed CLU expression in mutant p53 human HaCaT keratinocytes after vanadyl sulfate ($VOSO_4$) exposure and reported that apoptosis correlated with up-regulation of c-fos, decreased sCLU:nCLU ratios, and down-regulation of Bcl-2 levels and caspase activity. Overexpression of either c-fos or v-fos induced the same responses in HaCaT cells. Interestingly, sCLU overexpression failed to protect HaCaT cells from $VOSO_4$, but nCLU overexpression induced apoptosis. Dr. Saverio Bettuzzi (University of Parma, Parma, Italy) measured the half-life of sCLU at <2 h, and its turnover was tightly regulated by ubiquitin proteasome–mediated degradation.

CLU as a Modifier of Tumor Suppression

Several investigators presented evidence of the potential role of CLU as a tumor suppressor in several animal and cell models. Dr. Saverio Bettuzzi found that small interfering RNA (siRNA)–mediated sCLU knockdown induced cell cycle progression, with elevated proliferative markers. He also reported that TRAMP mice, carrying a probasin-driven SV40 T antigen, had highly aggressive tumor development when crossed with CLU knockout ($CLU^{-/-}$) mice. Onset of cancer was earlier, with higher numbers of primary tumors and elevated, poorly differentiated metastatic tumors per mouse, in comparison with wild-type TRAMP mice. Thus, loss of CLU seemed to drive tumor progression, suggesting that CLU might be a tumor suppressor. In support of this view, Dr. Arturo Sala (Institute of Child Health, London, United Kingdom) showed that full-length sCLU mRNA was down-regulated in neuroblastomas associated with MYCN gene amplification and presented direct evidence that N-MYC suppressed sCLU expression. The penetrance of neuroblastomas in N-MYC–transgenic mice was significantly increased after deletion of one or both sCLU alleles, and sCLU siRNA-transduced neuroblastoma cells acquired increased metastases when xenografted into mice. Ablation of CLU was accompanied by nuclear factor-κB (NF-κB) activation in developing neuroblastomas and during epithelial to mesenchymal transition (EMT). To corroborate that CLU inhibits NF-κB, Dr. Gilles Chiocchia (Cochin Institute, Paris, France) showed that specific sCLU subfragments bound and stabilized IκB-α, resulting in NF-κB inhibition. Dr. Andrei

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

The 5th International Clusterin/Apolipoprotein J Workshop was held in Spetses, Greece, June 2–5, 2008, and cochaired by Drs. Efstathios S. Gonos and Ioannis P. Trougakos. A complete listing of speakers and presentation titles is in a supplement. Prof. Saverio Bettuzzi (saverio.bettuzzi@unipr.it) will organize the "6th International Clusterin/Apolipoprotein J workshop" in Parma, Italy.

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Thomas-Tikhonenko (University of Pennsylvania, Philadelphia, PA) reported that Myc-transformed epithelial cells down-regulated sCLU and that sCLU could inhibit cell growth *in vitro* and prevent carcinogenesis *in vivo*. He further reported that Myc up-regulated microRNAs, specifically the miR-17-92 cluster that apparently silences sCLU gene transcription, and confirmed that miR-17-92 blockade can restore sCLU expression to impede tumor cell growth. Thus, Myc down-regulation of sCLU may be critical for cell transformation.

CLU as a Modifier of Tumor Promotion

In contrast, sCLU also possesses oncogenic properties by its ability to enhance cell proliferation and inhibit apoptosis. Dr. Sabina Pucci (University of Rome, Rome, Italy) evaluated sCLU levels in sera and stools of healthy donors versus colorectal cancer patients. A relationship between interleukin-6 (IL-6) and sCLU levels was found, correlating with progressive disease. Screening of both molecules was proposed *in situ* with circulating prognostic markers for noninvasive colorectal cancer. Additionally, during colon carcinoma progression, IL-6 and vascular endothelial growth factor (VEGF)-A production influenced tumor cell proliferation, favoring apoptotic escape and cell migration by interfering with Ku70-CLU-Bax interactions. It was shown *in vitro* that these interactions were modulated by IL-6. Moreover, IL-6 together with VEGF inhibited Bax-dependent cell death, promoted cell survival and tumor invasion, and increased the production of sCLU, thus shifting death to survival. Dr. Bon-Hong Min (Korea University, Seoul, Korea) reported that sCLU stimulated astrocyte proliferation through epidermal growth factor receptor (EGFR)–mediated extracellular signal-regulated kinase (ERK) activation. Knockdown of sCLU via siRNA or ERK inhibition suppressed growth, whereas sCLU overexpression or exogenous treatment with purified sCLU promoted EGFR phosphorylation at Y1173 and enhanced astrocyte proliferation. Dr. Amina Zoubeidi working with Dr. Martin Gleave (University of British Columbia, Vancouver, British Columbia, Canada) showed that elevated sCLU levels promoted prostate tumor cell survival by increasing NF-κB nuclear translocation and transactivation. Significantly, sCLU acted as an ubiquitin-binding protein that enhanced COMMD1 and I-κB proteasomal degradation via interaction with E3 ligase family members. For confirmation, sCLU knockdown was shown to stabilize COMMD1 and I-κB, blocking NF-κB release, whereas sCLU levels positively correlated with expression of known NF-κB–regulated gene signatures. Dr. Ioannis P. Trougakos (Institute of Biological Research and Biotechnology, Athens, Greece) described changes in signal transduction after the singular loss or overexpression of sCLU in nonstressed human cells, as well as changes in sCLU levels on aging. Depletion of sCLU signaled stress-inducing, p53-dependent growth retardation, altered proapoptotic to antiapoptotic Bcl-2 protein ratios, and disrupted Ku70-Bax complexes. Bax-activated mitochondrial dysfunction and elevated apoptosis ensued because sCLU functions as a cytosol retention factor for Bax. Moreover, he reported that gene expression signatures induced in response to altered sCLU expression levels included cell cycle, focal adhesion, p53, insulin, mammalian target of rapamycin, and actin cytoskeleton signaling pathways. Intriguing data were presented by Dr. Claudia Koch-Brandt (University of Mainz, Mainz, Germany), linking Toll-like receptor 3 (TLR3) with transcriptional activation of sCLU, leading to cytoprotective and anti-inflammatory responses. In addition to polyinosinic acid:poly-CMP that is a ligand for TLR3, CLU gene activation was also stimulated by purified cellular RNA and RNA released from necrotic cells.

Regulation of sCLU Expression

In support of the prosurvival and tumor-promoting role of sCLU, Dr. David A. Boothman (University of Texas Southwestern Medical Center, Dallas, TX) reported an insulin-like growth factor-I (IGF-I)–mediated sCLU induction pathway (7). The ATM (mutated in ataxia telangiectasia) kinase, induced by low doses of irradiation or DNA double-strand break–inducing agents, activated IGF-I production that, in turn, induced sCLU gene transcription. Importantly, all genetically unstable cells analyzed to date (i.e., $H2AX^{-/-}$, $MDC1^{-/-}$, $NBS1^{-/-}$, $MMR^{-/-}$, and $mTR^{-/-}$) showed elevated basal ATM-IGF-I/IGF-IR-sCLU expression. This pathway was exquisitely regulated by p53 in the cell, whereby IGF-I promoter transactivation was repressed by p53-NF-Y interactions. Dr. Boothman also elaborated on the role of transforming growth factor-β1 (TGF-β1) in sCLU induction. In contrast to irradiation, where p53 could repress IGF-I–mediated sCLU expression, TGF-β1–treated wild-type p53 cells induced expression of this axis. Molecularly, TGF-β1–induced Hdm2 gene transactivation, in conjunction with AKT overexpression/activation and p-Hdm2–dependent p53 degradation, stimulated IGF-IR/SRC/mitogen-activated protein kinase (MAPK)/ERK signaling, causing Egr-1–mediated sCLU expression. Using sCLU knockdown, Dr. Boothman showed that a positive feedback loop for sCLU expression played a role in survival and EMT transition in human colon and non–small cell lung cancers. He proposed that IGF-IR/SRC/MAPK/ERK/Egr-1 signaling was a universal regulatory pathway for the expression of this gene.

Dr. Efstathios S. Gonos (Institute of Biological Research and Biotechnology, Athens, Greece) reviewed the involvement of sCLU in aging. Although sCLU mRNA and protein levels were up-regulated *in vitro* during either replicative- or stress-induced premature senescence of primary human cells as well as *in vivo* during normal aging, serum levels were lower in healthy centenarians versus elderly donors. He proposed that sCLU was central to cellular homeostasis and that during aging sCLU up-regulation occurred in response to chronic stress. Dr. Boothman then showed data linking IGF-I in replicative and stress-induced sCLU induction. Dr. Julie Y. Djeu (H. Lee Moffitt Cancer Center, Tampa, FL) analyzed docetaxel resistance in androgen-independent PC3 and DU145 prostate tumor cells and found elevated signal transducer and activator of transcription 1 (STAT1) and sCLU levels versus parental tumor cells. STAT1 knockdown via siRNA prevented sCLU gene and protein expression while restoring docetaxel sensitivity. Thus, STAT1 controlled sCLU production, leading to docetaxel resistance. Moreover, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) resistance was also traced to CLU gene expression. Expression of sCLU was positively regulated by SRC/Janus-activated kinase (JAK)/STAT signaling and inhibition of SRC/JAK1 kinases by resveratrol abolished sCLU expression and resensitized cells to TRAIL. Thus, resveratrol may be added to current chemotherapeutic regimens to treat prostate cancer, circumventing docetaxel or TRAIL resistance.

Chaperone Activity—Neurologic Diseases

Another major function of CLU is its chaperoning capacity for protein stabilization and facilitation of clearance of damaged

(unfolded) proteins. Dr. Mark Wilson (University of Wollongong, Wollongong, New South Wales, Australia) identified putative endogenous extracellular ligands for the chaperone action of sCLU in human plasma. CLU-misfolded protein complexes were of high molecular weight (>4 × 10^7 Da) and could be traced *in vivo* for rapid clearance in the liver and spleen. Putative receptors for these complexes were identified in rat hepatocytes. Thus, CLU may exert a key role in clearing damaged proteins from extracellular spaces and protect the body from potentially dangerous accumulation of aggregated proteins and deposition, such as in Alzheimer's disease. Dr. Jeong Hun Kim (Seoul National University, Seoul, Korea) showed that sCLU played a critical function in retinal vessel development as a secreted molecular chaperone. This isoform was cytoprotective in ischemia-induced retinal endothelial cell apoptosis and in diabetic retinas *in vitro*. Analysis of mechanism indicated that sCLU had antipermeable activity against high-glucose or glycation end product–induced tight junction breakdown. Because prior data from this and other groups indicated similar functions in cancer cells, sCLU may play a role in oxidative high-glucose use (i.e., Warburg effect) and regulate tumor cell metabolism. This idea seemed consistent with the IGF-I mediated regulation of sCLU.

Insight into the Dual Role of CLU as a Tumor Suppressor and a Tumor Promoter

At the workshop, the idea began to emerge that the dual nature of CLU as a tumor suppressor and a promoter may not be contradictory. The outcome may depend largely on the isoform available, on the time and context that CLU is expressed in the life of a cell, and the microenvironment. In terms of sCLU, including its presecretory form, it can be speculated that a normal cell, on exposure to oxidants or DNA-damaging agents, will require sCLU to bind misfolded proteins until they can be properly refolded or recycled through the ubiquitination pathway for degradation. Thus, without sCLU, as in the case of $CLU^{-/-}$ mice, the cell can accumulate damaged proteins, some of which may be critical for cell homeostasis, and result in transformation. Under this scenario, sCLU acts as a tumor suppressor. However, when tumor initiation has already occurred or when the tumor cells are being treated with toxic chemotherapeutic agents, cell survival is paramount for tumor maintenance. In this case, tumor cells must have sCLU to protect against DNA-damaging agents and sCLU thus becomes a critical component for cell survival. In addition, growth factors necessary for tumor cell proliferation, such as IL-6, VEGF, IGF-I, and EGF, as well as tumor-derived factors, such as TGF-β1, can stimulate sCLU production that can then mediate tumor or senescent cell survival. In this latter case, sCLU within a transformed cell acts as an oncogene. These concepts are relatively unexplored but are easily testable. In terms of nCLU, it is invariantly proapoptotic and it is possible that a balance of sCLU/nCLU in a cell dictates its fate. Interestingly, nCLU is often absent in advanced tumors or tumor cell lines.

Suggested Terminology for CLU Gene Locus Protein Isoforms

Previously known by various names, including testosterone repressed prostate message-2, sulfated glycoprotein-2, apolipoprotein J, and X-ray–inducible transcript #8, CLU was designated the universally accepted name for this gene at the 1st International CLU workshop. At this workshop, a unifying terminology for the various CLU protein isoforms was adopted based on the two characterized CLU gene transcripts. The main gene transcript identified from the CLU gene locus (Supplementary Fig. S1*A*), sCLU isoform 2 (National Center for Biotechnology Information: NM_203339.1; predicted molecular weight, 50.1 kDa) is detected as an ~60-kDa glycosylated presecretory form of sCLU and should hereafter be referred to as psCLU (Supplementary Fig. S1*B*). Once it is cleaved and heavily glycosylated to form the mature, secreted heterodimeric ~75- to 80-kDa protein (Supplementary Fig. S1*C*), it should be referred to as sCLU. The sCLU protein form is thus composed of sCLUα and sCLUβ chains that are of ~40 kDa (Supplementary Fig. S1*B* and *C*). The second CLU gene transcript lacks the endoplasmic reticulum–targeting sequence at exon 2 (nCLU; Supplementary Fig. S1*A*) and its product is detected as an ~49-kDa nonglycosylated precursor nCLU protein (pnCLU; predicted molecular weight, 48.8 kDa) in the cytosol and an ~55-kDa glycosylated protein (referred to as nCLU) in the nucleus.

Future Directions

CLU expression changes are a sensitive indicator of oxidative stress in mammalian cells (8) and may play a role in genomic instability (9–11) seen during tumor progression (1–3) and in pathologic conditions associated with deposition of protein aggregates (e.g., Alzheimer's disease, inflammation, stroke, and heart attacks; ref. 8). Its function as a molecular chaperone in human serum and its ability to exist in two forms, sCLU to mediate cell survival and nCLU to induce apoptosis, endow it with unique properties that can decide the fate of a cell. Results suggest that IGF-I-SRC-ERK signaling, even in response to TGF-β1 exposure, may be key regulators of sCLU expression, accounting for tumor progression and other chronic diseases. Interestingly, Dr. Ofer Levy (Compugen Ltd., Tel Aviv, Israel) presented data for a peptide that specifically bound sCLU and caused growth arrest as well as synergistic cytotoxicity with Taxol against breast, lung, prostate, and melanoma cancer cells. Thus, sCLU may be a "druggable target" for tumor therapy. What is sorely needed is research illuminating roles for sCLU versus noninducible nCLU in normal and pathologic tissues. Understanding the molecular regulation and structure of CLU isoforms is essential, especially given that the ratio of sCLU:nCLU seems critical for cancer survival and progression. It is essential to address cell- and tissue-specific CLU functions during tumor initiation and progression. Further research into CLU functions and downstream signaling pathways affected by CLU expression is also clearly needed. Collaborations between groups are needed to enhance clinical opportunities to exploit these findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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